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RESEARCH ARTICLE

The microanatomy of the central nervous system and brain of the Indo-Pacific seahorse, *Hippocampus barbouri*, during development

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ABSTRACT. The central nervous system (CNS) of Teleostei is a complex system of self-governance and its morphology is reflected in the physiological and reproductive behaviors. The Indo-Pacific seahorse, *Hippocampus barbouri* Jordan & Richardson, 1908, is a new candidate species for aquaculture in Thailand. In this study, we investigated the brain morphology of *H. barbouri* across various developmental windows. Light microscopic observations of adult brains revealed a large optic tectum in the mesencephalon, whereas the cerebral hemispheres and the cerebellum are of medium size. The detailed brain structures were generally similar to those of other teleosts; however, only five distinct layers were present in the optic tectum, including the stratum marginale, stratum opticum, stratum album central, stratum griseum central, and stratum periventriculae, versus six layers observed in other fish. One day after birth (1 DAB) the brain was a packed structure without any clear sub-structures. The number of capillaries in the optic tectum began to increase at 6 DAB, and at 14 DAB several features, including small blood vessels in the optic tectum and Purkinje cells, became noticeable. By 35 DAB, the optic tectum became highly vascularized and included five layers. Additionally, large Purkinje cells were developed in the cerebellum. Based on the brain development pattern, we speculate that the predatory ability of this fish starts to develop from 6 to 14 days after birth.

KEY WORDS. Histology, seahorse, spinal cord, Thailand

INTRODUCTION

The central nervous system (CNS) integrates the information from sensory organs and mediates the response to environmental stimuli, whereas the spinal cord controls locomotion independently of the brain (Genten et al. 2009). This is a general pattern in animals, including teleosts (Northcutt and Braford 1980, Nieuwenhuys and Meek 1990, Yamamoto 2008, Senarat et al. 2016), and many studies have demonstrated that the brain morphology has adaptive significance and influences behavior and habits (Kotrschal et al. 1998, Gonzalez-Voyer and Kolm 2010, Park and Bell 2010). For example, the large cerebral hemisphere is associated with a higher degree of learning, sensory integration, and spatial navigation (Gonzalez-Voyer and Kolm 2010, Park and Bell 2010), while a large and optic tectum is associated with good vision and orientation response (Huber et al. 1997, Pollen et al. 2007). Brain morphology investigations therefore elucidate the physiology and behavior of fish.

It is well-known that the reproduction of teleosts is controlled by the hypothalamic-pituitary-gonadal axis (HPG axis) (Nagahama 2000). In this axis, the hypothalamus, a major area of diencephalon, releases hypothalamic hormones, especially gonadotropin releasing hormone (GnRH), to control the synthesis and secretion of the pituitary gonadotropic hormones (gonad-

otropins, GTHs): GTH I (FSH-like) and GTH II (LH-like). These gonadotropic hormones are essential for gonadal development and maturation as well as stimulation of gametogenesis in several species of fish (King and Millar 1992, Sherwood et al. 1993, Feist and Schreck 1996, Senarat et al. 2019a). There is an increasing interest in locating these reproductive hormones in the brain using immunocytochemistry and immunofluorescence (Naito et al. 1991, Nyuji et al. 2011, Senarat et al. 2019b), which will be useful in the assessment and control of gonadal differentiation of fish (Murata et al. 2012). However, to interpret the results detailed anatomical information on the brain is required. Therefore, the accumulation of neuroanatomical knowledge will be also significant for the development of evidence-based aquaculture.

The Indo-Pacific seahorse *Hippocampus barbouri* Jordan & Richardson, 1908 (Syngnathidae) is an economically important fish. This fish has been reared at the Phuket Biological Center, Thailand. The next step to broaden the stock of this fish is to increase its sustainable production with appropriate management. Scientific reports on the reproductive biology of this seahorse species is still limited (Nur et al. 2016, Kamnurdnin 2017), and more importantly no neuroanatomical studies have been reported. This study aims to provide the baseline information on the structure and development of the CNS of *H. barbouri* in captivity. To this end, *H. barbouri* were subjected to the histological observation from 1 to 35 days after birth (DAB).

MATERIAL AND METHODS

Hippocampus barbouri reared in a standard culture system of the PMBC, Thailand, were used for the observation. We collected samples of juvenile (1, 6, 12, 14 and 24 DAB) and adult (35 DAB) stages (n = 3 for each DAB) from October to December, 2017. Kamnurdnin (2017) studied the effects of food on the growth and gonadal development of this fish, and we used the brains of his specimens in this study. Information on the samples are shown in Table 1. All specimens were acclimatized for about 14 days in shaded concrete tanks filled with sea water at 26–28 °C, salinity level of 31–33 ppt, and photoperiod of 12:12 hours light-dark. The fish were fed wild krill twice a day. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University (Protocol Review #1623004).

Table 1. Size and number of captive *Hippocampus barbouri* samples used in this study.

Seahorse stages	Days after birth (DAB)	Numbers	Total length (mm)
Juveniles	1	3	15.6 ± 0.78
	6	3	20.5 ± 1.04
	12	3	35.2 ± 2.22
	14	3	43.2 ± 2.56
	24	3	48.3 ± 2.43
Adults	35	3	58.2 ± 3.65

The fish used in the experiment were euthanized by the rapid cooling method (original protocol by Wilson et al. 2009) and then fixed overnight in a solution containing Davidson's fixative (Dietrich and Krieger 2009) at room temperature. After dissection, the anatomical features of the whole brain were examined from various views (dorsal, longitudinal and ventral), and cross sections of the mid-body (at 35 days) were observed under the SZX12 stereomicroscopy (Olympus, Japan). Photographs were taken with an Olympus DP 11 digital camera. The major anatomical structures were subjected to a morphometric analysis (corpus cerebelli length, corpus cerebelli width, telencephalon width, tectum opticum length, lobus inferior hypothalami length, lobus inferior hypothalami width, cerebellum length, cerebellum width and vagal lobe length) following the standard guideline from Abrahão and Shibatta (2015). All morphometric parameters were measured using an automated cellular image analysis system, Digimizer software, version 3.7.0. Schematic diagrams were drawn using the Adobe Illustrator CS5.

To examine the CNS structure, all brain regions, including the spinal cord of all samples (1, 6, 12, 14, 24 and 35 DAB), were processed using a standard histological technique (Presnell and Schreibman 2013, Suvarna et al. 2013). The paraffin blocks were crossly and longitudinally sectioned at a thickness of 4 µm and stained with Harris's hematoxylin and eosin (H&E). All histological sections were examined for the CNS structure, whereas brain development was assessed by comparing images from 1 to 35 DAB taken by the TE750-Ua camera (Leica, Heidelberg, Germany).

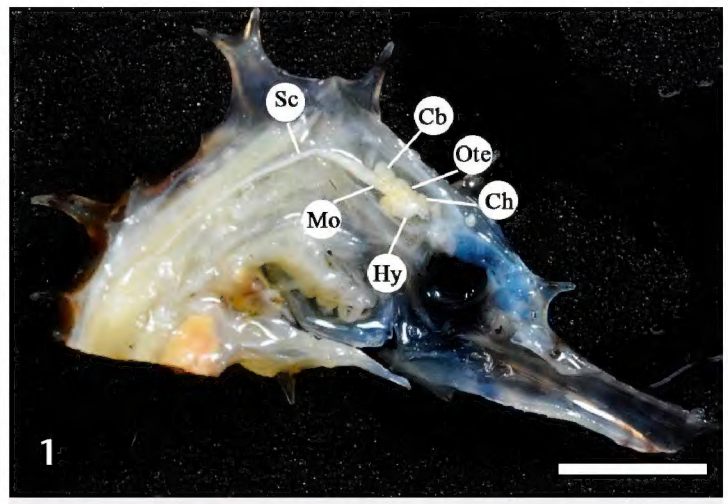
RESULTS

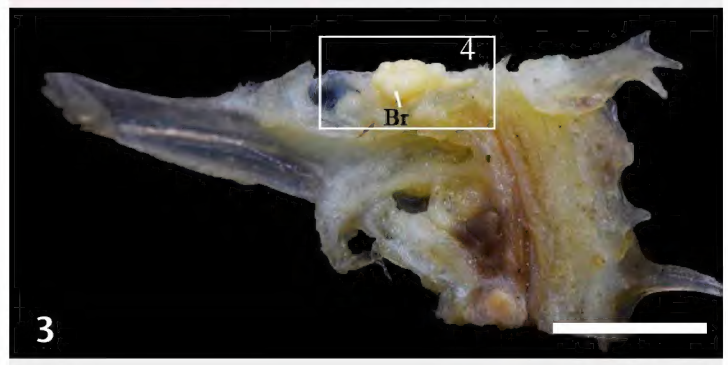
Gross anatomy and morphometric analysis of the brain

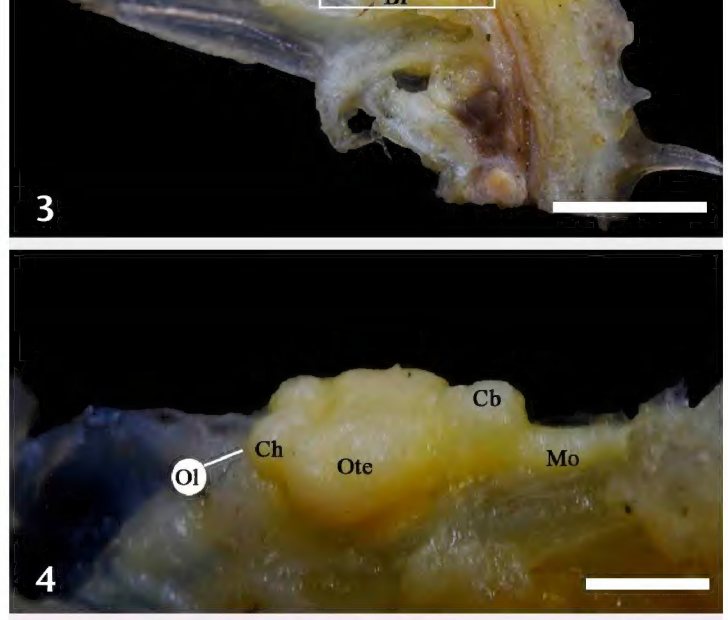
The CNS of *H. barbouri* was composed of the brain and spinal cord (cerebrospinal system; Figs 1–3). In the longitudinal view, cerebral hemisphere, optic tectum, cerebellum, hypothalamus and modular oblongata were clearly observed and morphometric data are shown in Table 2. The olfactory lobes were seen anteriorly from the cerebral hemisphere and optic tectum (Figs 4, 5). The optic tectum was apparently the largest area (Figs 6–8, Table 2), followed by the cerebellum and cerebral hemisphere located anteriorly and posteriorly from the optic

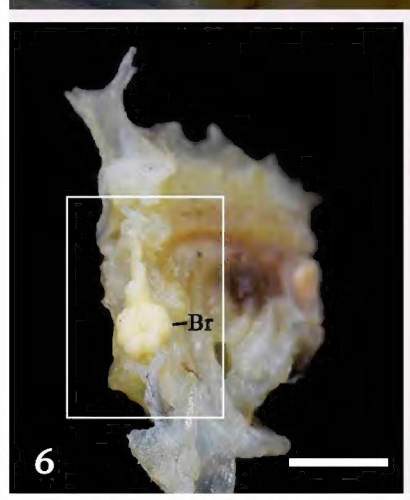
Table 2. Morphometric analysis of brain on *Hippocampus barbouri* at 35 DAB.

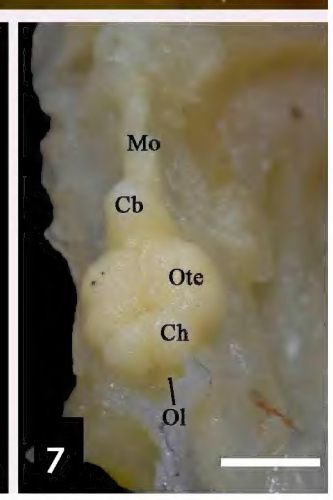
Brian regions (n = 3)	Mean (µm) ± SD
Corpus cerebelli length	277.40 ± 0.87
Corpus cerebelli width	256.54 ± 0.96
Telencephalon width	781.73 ± 1.02
Tectum opticum length	1220.40 ± 1.12
Cerebellum length	714.34 ± 1.20
Cerebellum width	503.21 ± 0.97
Lobus inferior hypothalami length	610.68 ± 0.85
Lobus inferior hypothalami width	530.34 ± 0.95
Vagal lobe length	500.20 ± 1.16

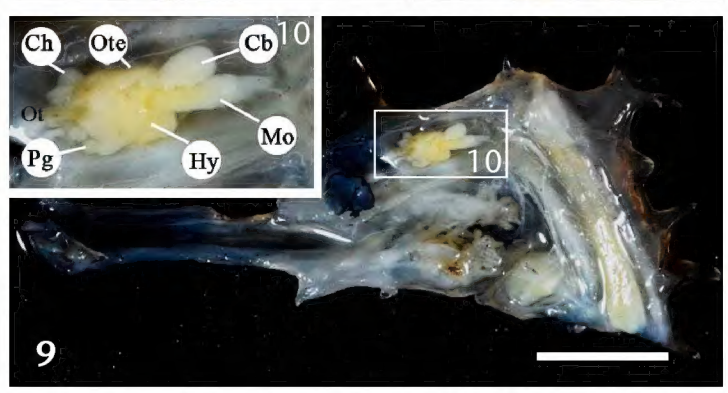


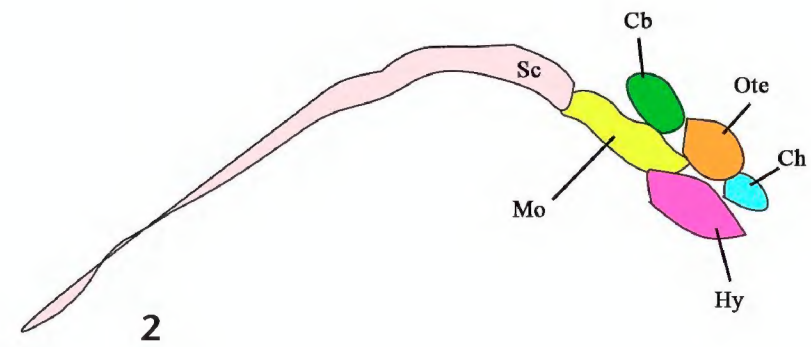


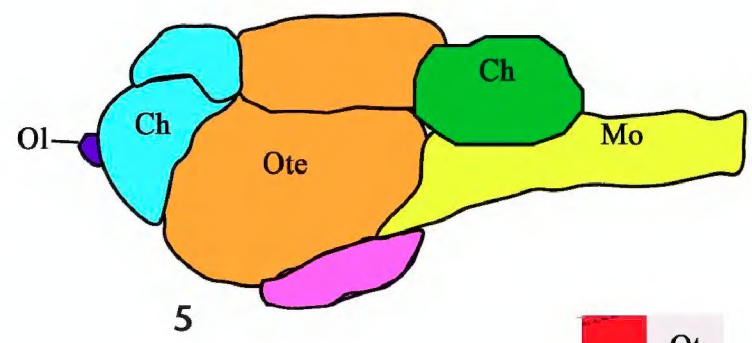


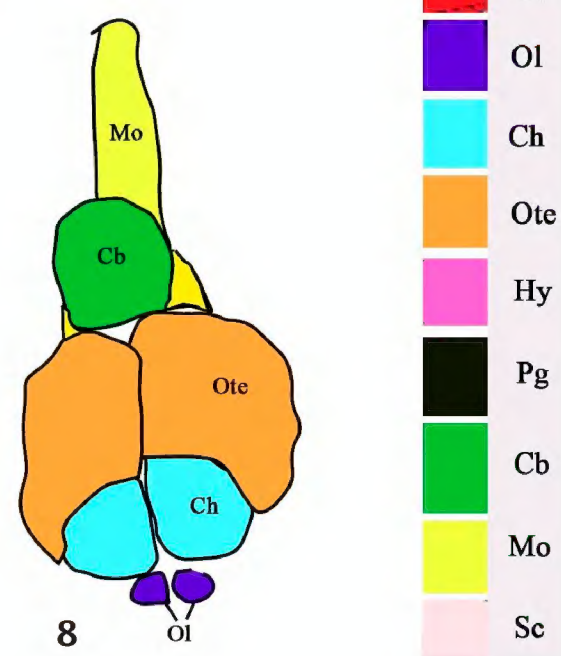


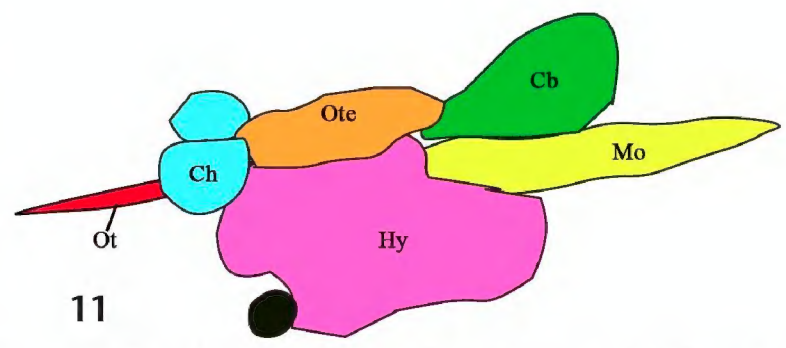












Figures 1–11. The central nervous system (CNS) of *Hippocampus barbouri* at 35 DAB. (1, 2) Morphology and schematic diagram of the CNS in a longitudinal view. The brain contained cerebral hemisphere (Ch), optic tectum (Otc), cerebellum (Cb), hypothalamus (Hy) and modular oblongata (Mo). The spinal cord (Sc) was also observed. (3) Morphology of the brain in lateral view. (4, 5) Morphology and schematic diagram of the brain at high magnification. The olfactory lobe (Ol), Ch, Otc, Cb and Mo were observed. (6) Brain morphology in dorsal view. (7, 8) Morphology and schematic diagram of the brain in dorsal view at high magnification.(9–11) Morphology and schematic diagram of longitudinal sections showing the olfactory tract (Ot), Ch, Otc, Cb, Hy and Mo. Scale bars: 1, 3, 6, 9 = 3 cm, 4, 7 = 0.5 cm.

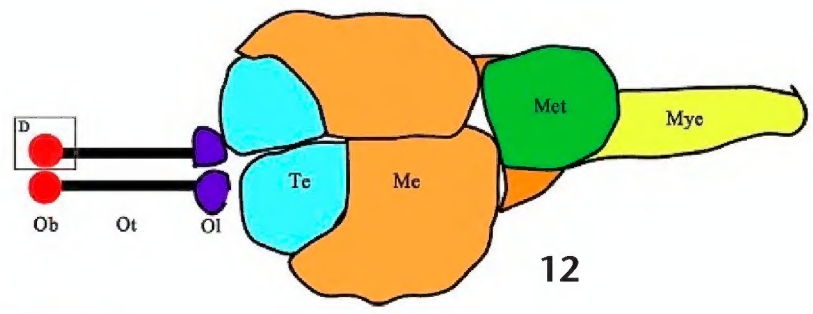
tectum, respectively (Figs 1–7, Table 2). The narrow medulla oblongata connected the brain and spinal cord (Figs 7, 8). In the lateral view, the olfactory tract was observed anterior to the cerebral hemisphere (Figs 10, 11).

Histological structure of the brain

According to the cellular composition, tissue architecture and localization, the brain was subdivided into five regions; telencephalon, mesencephalon, diencephalon, myelencephalon and metencephalon (Figs 12, 13). The olfactory bulbs were found in the nasal pit of the anterior region as a pair of elliptical solid sacs (Figs 12, 14, 15). The olfactory bulb was characterized by a surface consisting of ciliated sensory cells (or receptor cells) (Fig. 15). An oval nucleus with dark blue color was observed in the ciliated sensory cells (MT staining, Fig. 15).

Telencephalon. The telencephalon consisted of paired olfactory lobes and cerebral hemispheres (Fig. 12). The olfactory lobes were connected to the olfactory bulbs in the snout via the olfactory tract, a bundle of afferent nerves (Fig. 16). The cerebral hemispheres contained only neuroglia (or supporting cell) (Figs 17, 18), which were distinguished from neurons by their small nuclei surrounded by a thin acidophilic cytoplasm.

Mesencephalon. This region contained the optic tectum and is considered to be the main optic center involved in visual, auditory and lateral line processing. The optic tectum was separated from the epithalamus of the diencephalon by the third ventricle (Fig. 19). Histologically, this region was covered by a well-vascularized meninx primitiva. The mesencephalic aqueduct (sylvian aqueduct) connected the third and fourth ventricles. Five principal layers were recognized in the optic tectum (Fig. 20); from the outer to inner layers, tratum marginale; stratum opticum; stratum album centrale; stratum griseum centrale and stratum periventriculare. These layers had different cellular compositions in terms of neuronal density and afferent fiber connections.



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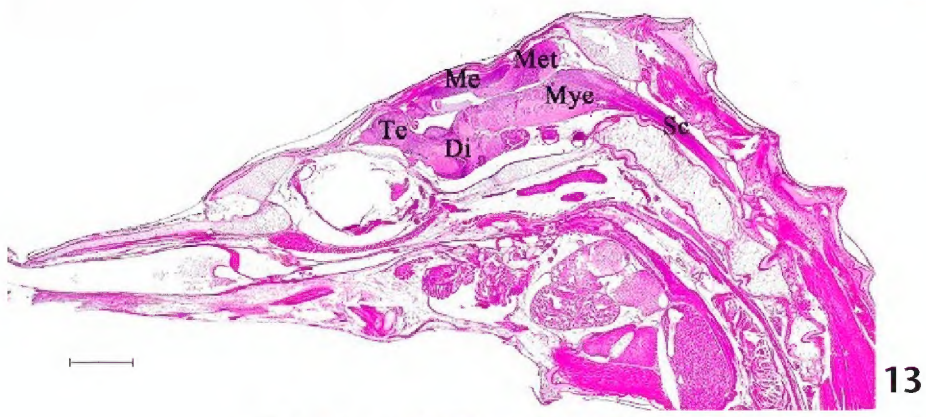

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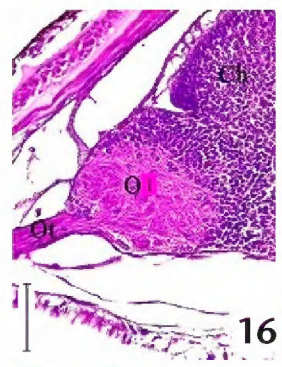

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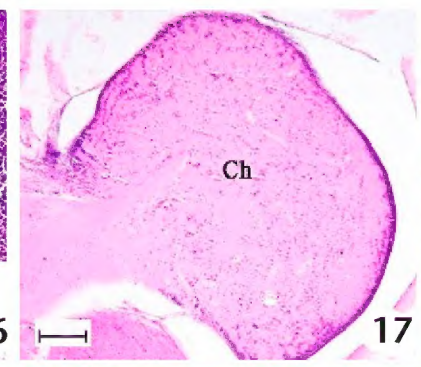

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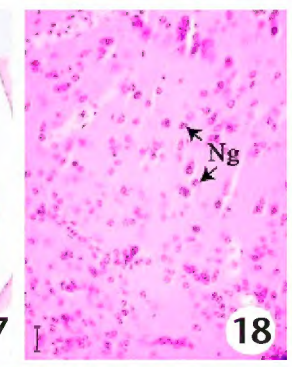

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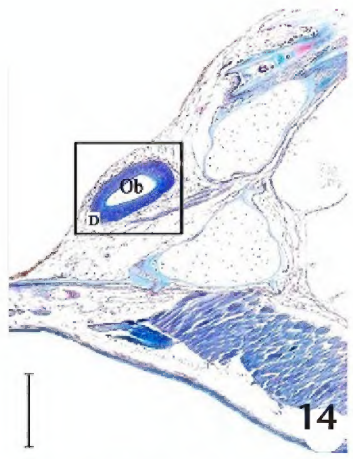

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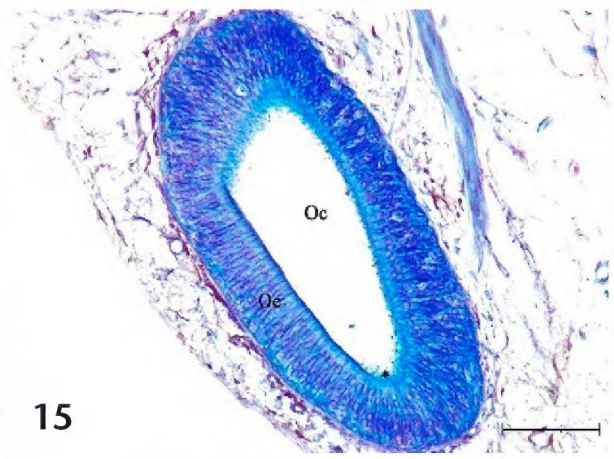

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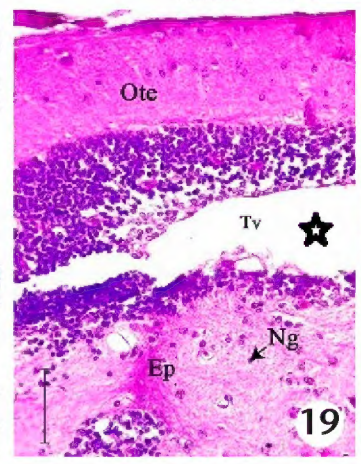

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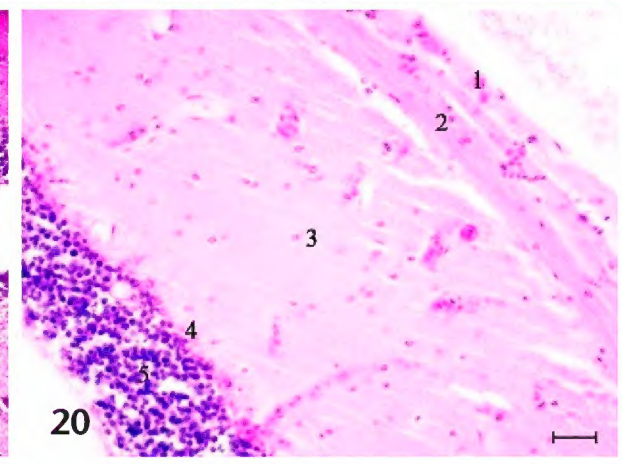

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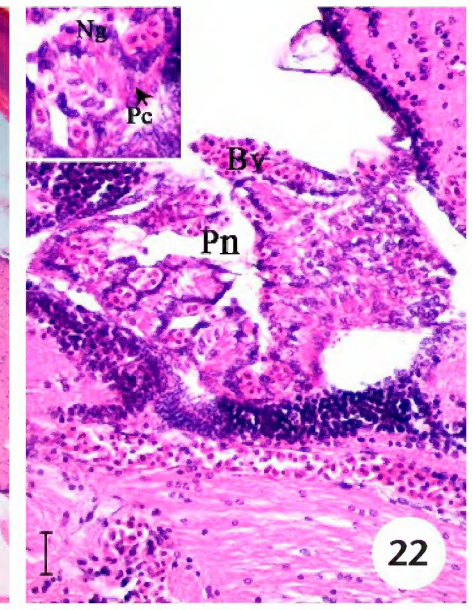
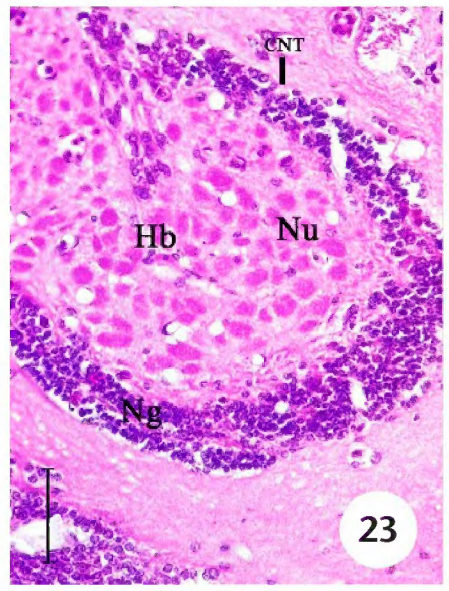
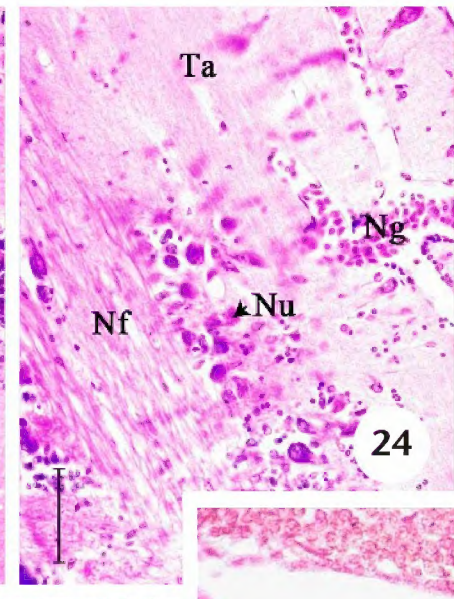
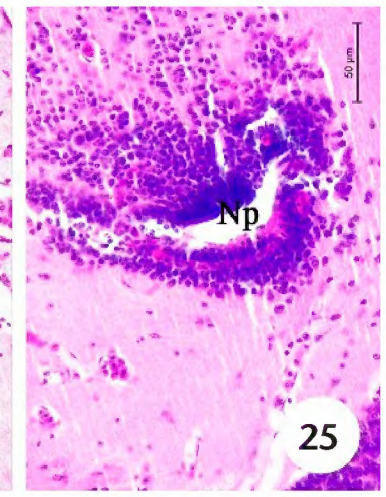
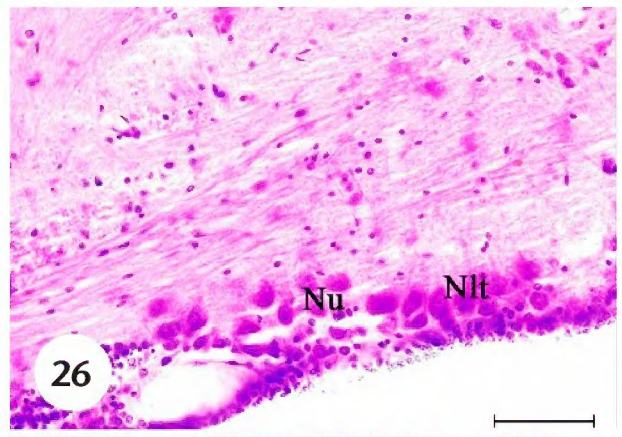
Figures 12–20. Schematic diagram and light micrograph of the brain of *Hippocampus barbouri* at 35 DAB. (12, 13) Overall brain structure in the dorsal view. Histological observation of the brain in the longitudinal section identified five regions including telencephalon (Te), mesencephalon (Me), diencephalon (Di), metencephalon (Met) and myelencephalon (Mye). Mye was connected to the spinal cord. (14) Location of the olfactory bulb (Ob) in nostril. (15) High magnification image of the olfactory bulb showing the olfactory cavity (Oc) surrounding with olfactory epithelium (Oe), olfactory cavity (Oc) and ciliated sensory cells with prominent cilia (*). (16) Olfactory lobe (Ol), olfactory tract (Ot) and cerebral hemisphere (Ch). (17, 18) Cerebral hemisphere (Ch) containing neuroglia. (19) Third ventricle (Tv) was found between the optic tectum (Otc) and epithalamus (Ep). (20) Histological classification of the optic tectum including 1= stratum marginale, 2 = stratum opticum, 3 = stratum album central, 4 = stratum griseum central and 5 = stratum periventriculae. Ng = neuroglia. Scale bars: 13 = 500 µm, 14 = 200 µm, 15, 16, 17, 19 = 50 µm, 20 = 20 µm.

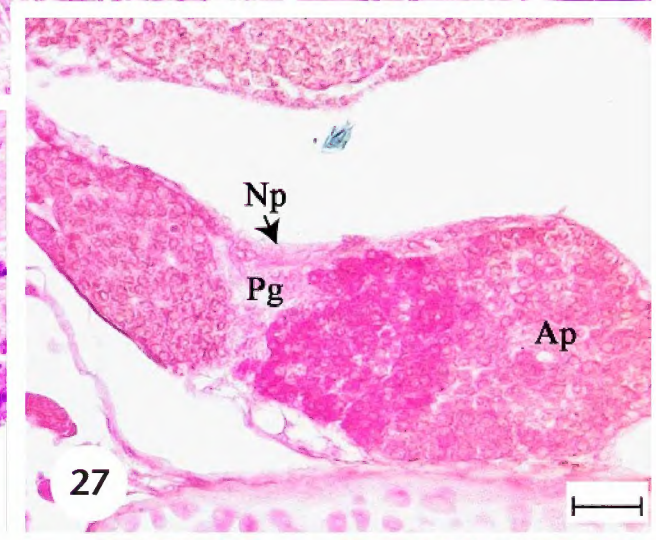


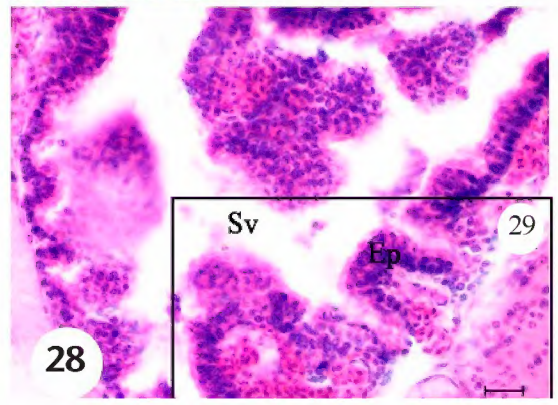


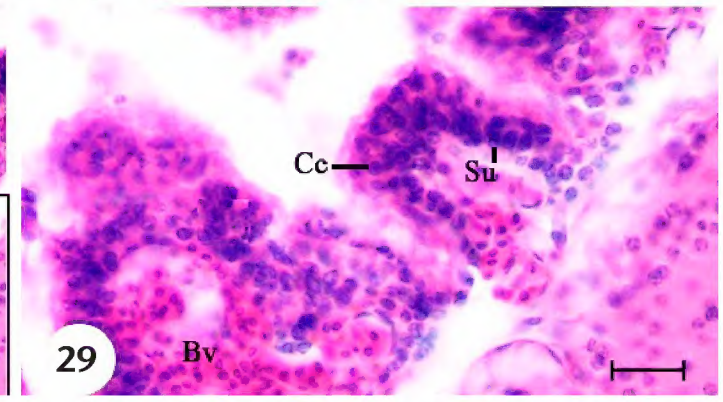












Figures 21–29. The diencephalon of *Hippocampus barbouri* at 35 DAB. (21) The diencephalon was subdivided into epithalamus (Ep), thalamus (Ta) and hypothalamus (Hy). (22) The pineal gland (Pn) contained blood vessels (Bv), pinealocytes (Pc) and neuroglia (Ng). (23) Habenula ganglion (Hb) was surrounded by a thin layer of connective tissue (CNT). It contained neurons (Nu) and neuroglia (Ng). (24) The Ta contained different cells including neurons (Nu) and neuroglia (Ng). Neuronal fibers (Nf) were also present. (25, 26) Several important regions of the hypothalamus including nucleus periventricularis (Np) and nucleus tuberalis lateralis (Nlt). (27) Two regions in the pituitary gland (Pg) included the neurohypophysis (Np) and the adrenohypophysis (Ap). (28–29) The succus vasculosus (Sv) was surrounded by the epithelium (Ep). Bv = blood vessel, Cc = coronet cell, Nu = neuron, Su = supporting glial cell. Scale bars: 22, 27, 28, 29 = 20 µm, 23, 24, 25, 26 = 50 µm.

Diencephalon. The diencephalon was located below the mesencephalon (Figs 13, 21). One of the main functions of the diencephalon was to receive the primary olfactory information from the telencephalon together with gustatory and optic information from other sections of the brain. The diencephalon is histologically divided into epithalamus, thalamus and hypothalamus (Fig. 21).

The epithalamus mainly contained the pineal gland (Fig. 22) and habenular ganglion (Fig. 23). The pineal gland contained at least two types of pineal parenchymal cells, pineal cells (or pinealocytes) and neuroglia (or supporting cells) (Fig. 22). A large basal nucleus was observed in the pineal cells. The neuroglia was generally found at the edge region of the pineal gland with an irregularly-shaped nucleus. The habenular ganglion was located close to the mesencephalon, being surrounded by a thin layer of connective tissue (Fig. 23). Several neurons were observed within the ganglion.

The thalamus was located between the epithalamus and the hypothalamus. In the thalamus we observed many nuclei of neurons and neuroglia (Fig. 24). Neuronal fibers (Nf) were also present.

The hypothalamus was the dominant region of the diencephalon, where the ventral diencephalon formed an infundibular structure (medial lobe) (Fig. 21). Several cell populations, including a large number of neurosecretory cells, were observed in the hypothalamus, with some important areas such as nucleus periventricularis and nucleus tuberalis lateralis (Figs 25, 26). Additionally, the pituitary gland (Fig. 27) and saccus vasculosus (Figs 28, 29) were extended from the hypothalamus. The pituitary gland contained two regions, neurohypophysis and adrenohypophysis. The saccus vasculosus was a small and capsule-like structure located in the caudal region of the hypothalamus. Histological evaluation showed that it is surrounded by a meninx primitiva, a highly vascularized endomeninx (Fig. 28). Extensive folds were observed in the epithelium (Fig. 28). Coronet cells with a basally located nuclei and supporting glial cells were attached to the epithelium (Fig. 29).

Metencephalon. The metencephalon contained the cerebellum (Fig. 30), and the posterior part of the cerebellum corpus was located below the optic tectum (Figs 32, 33). There were three layers including the outer molecular layer, a Purkinje cell layer and the inner granular layer of the cerebellum (Fig. 31). The outer molecular layer was basically comprised of dendritic fibers, whereas the Purkinje cell layer contained neurons, which form synapses with the dendrites (Fig. 31). The development of the inner granular layer was obvious with lining the principal neurons (Fig. 31).

Myelencephalon. The rostral region of the brain was the myelencephalon, comprising the paired vagal lobe and the medulla oblongata (Figs 32, 33). The vagal lobe was found in the dorsal medulla oblongata but was barely developed (Fig. 34). A large part of the medulla oblongata was comprised of vast neuronal bodies and a fossa rhomboidea (the anterior part of the fourth ventricle) in the caudal medulla oblongata (Fig. 34). Its ventricular wall was lined with the neuronal cell called "ependymal cell" (Fig. 35).

Histology of the spinal cord

The spinal cord extended from the myelencephalon to the vertebral column (Fig. 13). Neurons and neuroglia were considered to be the major component of the spinal cord (Figs 21–29); small and densely packed neurons were observed in the middle area of the spinal cord (Figs 36, 37). The central canal of the spinal cord was open to the fourth ventricle filled with the cerebrospinal fluid (CSF) (Fig. 35).

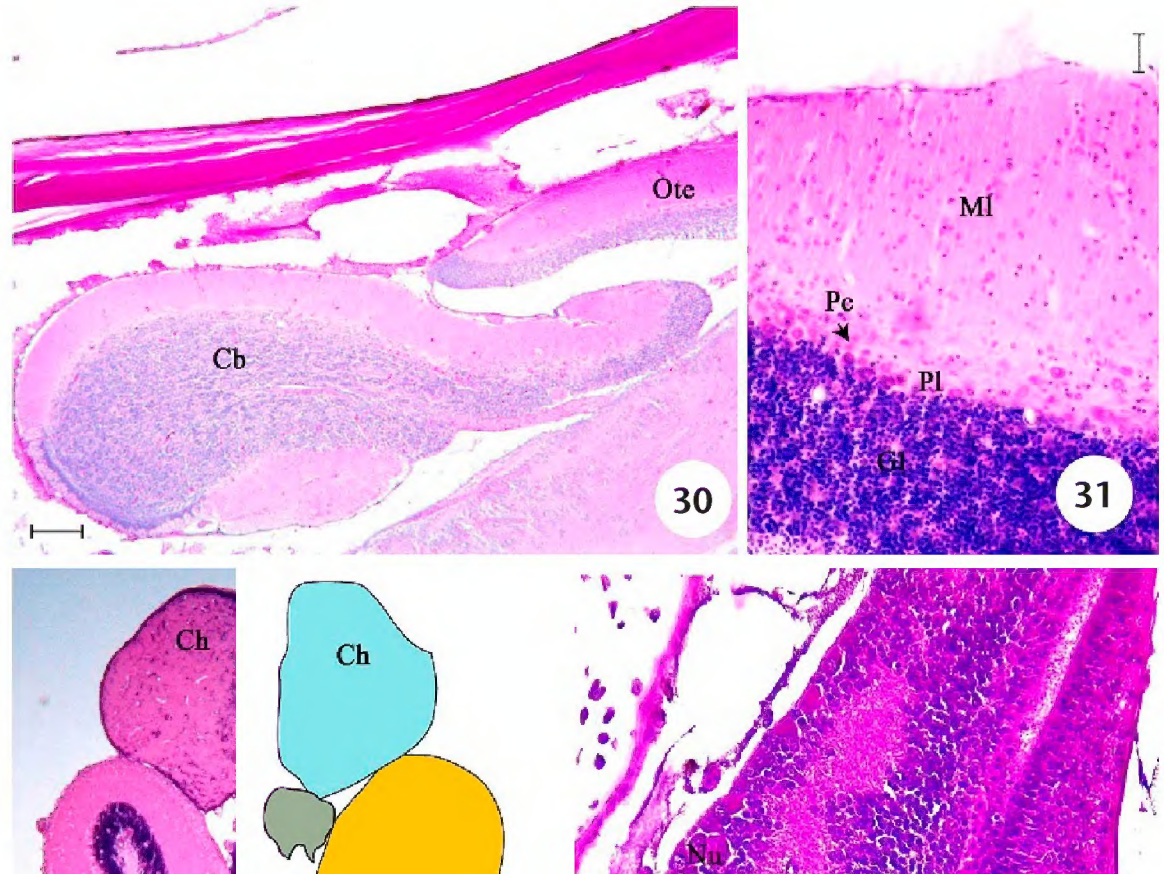
We also observed that the ganglion is a part of the nervous system outside the brain and spinal cord. Each ganglion had similar structure, containing a cluster of neural cell bodies, satellite cell (supporting cell) and neuronal fiber (Fig. 38).

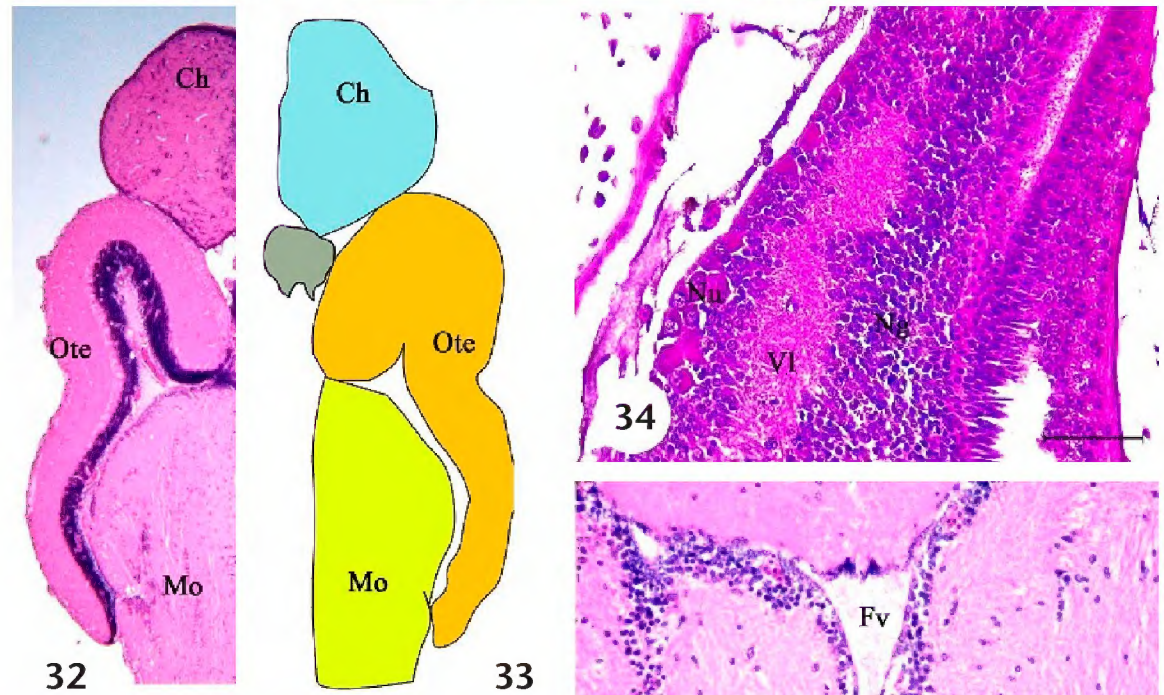
Development of the brain

Brain development patterns are shown in Figs 39–51. At 1 DAB, the brain was a packed structure without clear substructures, but it was still possible to be divided into telencephalon, mesencephalon, diencephalon, myelencephalon and metencephalon (Fig. 39). Both cerebral hemisphere and cerebellum were visible (Figs 40, 41); however, the Purkinje cells in the cerebellum were barely developed (Fig. 42). Blood vessels were hardly observed in the saccus vasculosus (Fig. 43). The number of capillaries began to increase at 6 DAB in the optic tectum (Fig. 44). At 14 DAB, several neuroglia were observed in the telencephalon (Fig. 45). Blood vessels started to form a network in the saccus vasculosus (Fig. 46) and the optic tectum (Fig. 47). Purkinje cells in the cerebellum were obviously developed by 14 DAB (Fig. 48). The pituitary gland was prominently composed of glandular tissues as positively stained in the PAS method (Fig. 49). At 35 DAB highly developed blood vessels and the five layers were clearly observed in the optic tectum (Fig. 50). The Purkinje cells became considerably apparent with many dendrites extending into the molecular layer (Fig. 51).

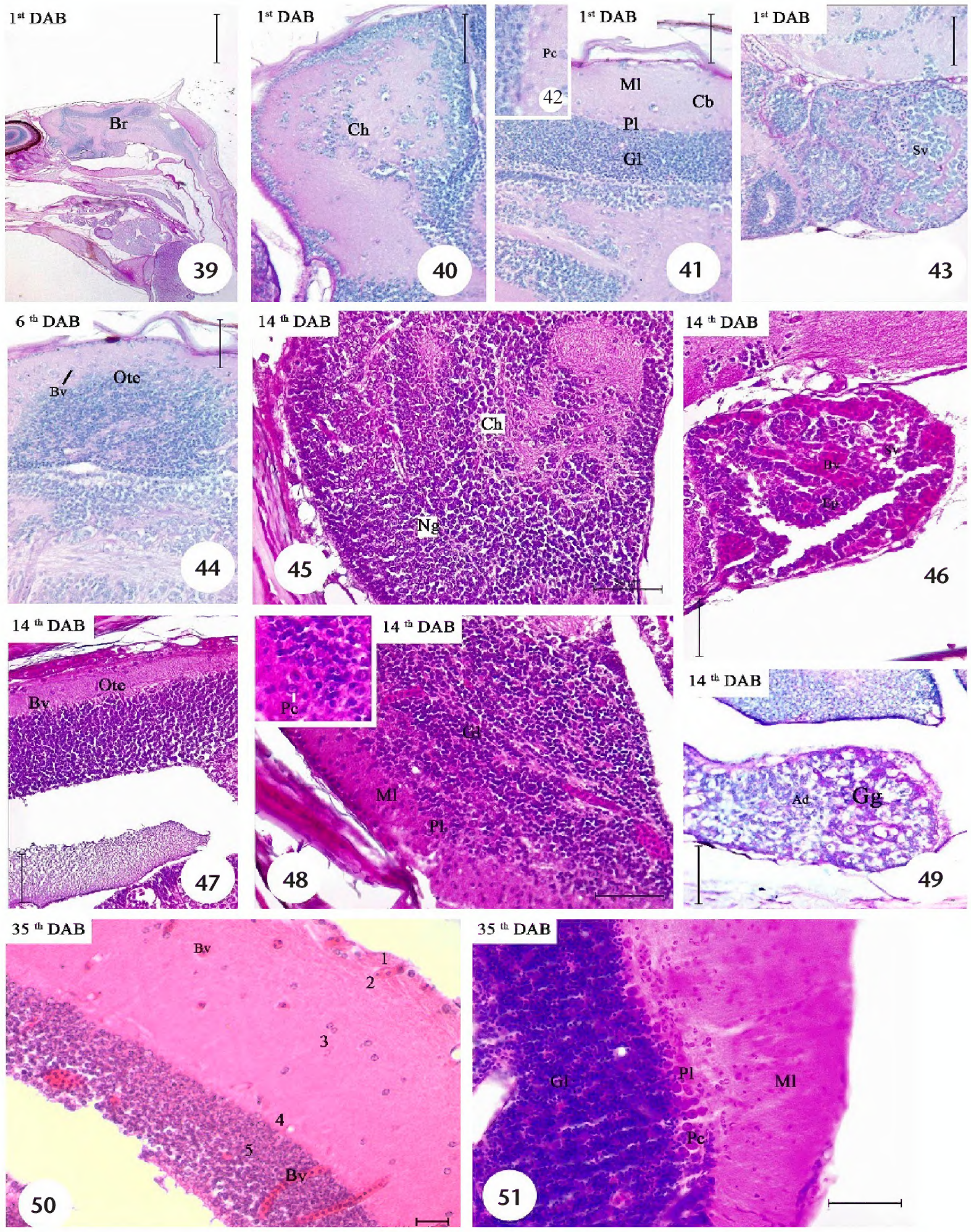
DISCUSSION

Studies in the field of evolutionary neuroscience have shown that the physiological and reproductive behaviors of a species are reflected in the structure of the CNS (Kotrschal et al. 1998, Nieuwenhuys et al. 1998, Gonzalez-Voyer et al. 2016, Tsuboi et al. 2017). We show that the brain of *H. barbouri* contains a large optic tectum, whereas the size of the cerebellum is not abnormal. Such structural characteristics have been associated with a high-level sensory integration and spatial navigation (Gonzalez-Voyer and Kolm 2010, Park and Bell 2010), visual-tactile information and orienting response (Huber et al. 1997, Pollen et al. 2007). It is also associated with feeding on fast-moving prey (Kotrschal et al. 1998) because the optic tectum is involved in multisensory integrations of visual signals with sensory informa-





Figures 30–38. The mylencephalon and metencephalon of *Hippocampus barbouri* at 35 DAB. (30) The cerebellum was found behind the optic tectum (Ote). (31) High magnification image of the cerebellum layers including the outer molecular layer (MI), Purkinje cell layer (Pl) and the inner granula layer (Gl). The prominent Purkinje cells (Pc) were observed. (32, 33) Structure and schematic diagram of the sagittal section that show the optic tectum (Ote) next to the medulla oblongata (Mo) of the myelencelphalon. (34) Vagal lobe (Vl) in the myelencephalon contained neuron (Nu) and neuroglia (Ng). (35) High magnification image showing that medulla oblongata is penetrated with the fourth ventricle (Fv). This region prominently contained neurons (Nu) and neuroglia (Ng). (36, 37) Cross section of the spinal cord (Cd) was observed, which high magnification of the accumulated neuron (Nu) was seen. The central canal (Cc) was lined by ependymal cell (Epc). (38) The ganglion (Gg) was connected with the dorsal or posterior root of the nerve fiber (Nf) originating from the spinal cord. It contained in both neuron (Nu) and satellite cell (Sac). Scale bars: 30 = 100 µm, 31, 35, 36, 37, 38 = 20 µm, 34 = 50 µm.



Figures 39–51. Light micrograph of *Hippocampus barbouri* brain development. (39) Packed structure of the brain at 1 DAB. (40) Cerebral hemisphere of the telencephalon at 1 DAB. (41) The cerebellum (Cb) contained the outer molecular layer (MI), Purkinje cell layer (Pl) and the inner granula layer (Gl). However, the Purkinje cells (Pc) were rarely observed in the Pl. (42) High magnification image of Pl where Pc were rarely developed. (43) The absence of the blood vessel in the saccus vasculosus. (44) Obvious development of the capillaries of the optic tectum at 6 DAB. (45) Increased neuroglia amount of the cerebral hemisphere. (46) Vascularized blood vessels in the saccus vasculosus. (47) Small blood vessels in the optic tectum. (48) Small Purkinje cells in the cerebellum. (49) Obvious development of glandular tissue (Gg) in the adrenohypophysis (Ad). (50) Optic tectum with highly developed blood vessels and the five distinct layers (1= stratum marginale, 2 = stratum opticum, 3 = stratum album central, 4 = stratum griseum central and 5 = stratum periventriculae). (51) Cerebellum containing Pc.(MI) Molecular layer, (GI) granular layer. Scale bars: 39 = 500 µm, 40, 41, 43, 44, 45, 46, 47, 48, 49, 51 = 50 µm, 50 = 20 µm.

tion from other modalities (Davis and Northcutt 1983, Bodznick 1991, Meek and Nieuwenhuys 1998). Although no information is available on the feeding ecology of this species during the first month of development, our finding probably reflects the structurally complex habitat where *H. barbouri* catches prey. This may be a unique characteristic for syngnathid fish since they commonly have the longest snout and consume highly mobile prey, such as mysids, shrimps and fish (Kotrschal et al. 1998, Kendrick and Hyndes 2005, Garamszegi et al. 2005, de Lussanet and Muller 2007, Van Wassenbergh et al. 2011, MacLean et al. 2014, Lefebvre et al. 2016, Tsuboi et al. 2017).

According to the histological observation, the brain of *H. barbouri* is subdivided into five regions: telencephalon, mesencephalon, diencephalon, myelencephalon and metencephalon, as generally observed in other teleosts (Northcutt and Braford 1980, Nieuwenhuys and Meek 1990, Genten et al. 2009, Yamamoto 2008, Senarat et al. 2016). The optic tectum of *H. barbouri* consisted of five layers, including the stratum marginale, stratum opticum, stratum album central, stratum griseum central, and stratum periventriculae. This result is not in line with previous observations of six layers in the optic tectum of *Rastreilliger brachysoma* (Bleeker, 1851) (Senarat et al. 2016) and other cyprinids as *Cyprinalla lulrensis* (Baird & Girard, 1853), *Notropis bairdi* Hubbs & Ortenburger, 1929 and *Notropis amabilis* (Girard, 1856) (Huber and Rylander 1991. Immunohistochemical observations, for example targeting the estrogen receptor and neurotrophin Trk receptor expression, will be needed to further test this feature.

An increase in the amount of blood vessel in the saccus vasculosus of *H. barbouri* happened at 14 DAB. As reported by Nakane et al. (2013), the saccus vasculosus is a complex organ and functions as a seasonal sensor by recognizing the photoperiods. Dammerman (1910) claimed that capillaries are responsible for nutritive substances that significantly affect the function of the saccus epithelium. On the other hand, Purkinje cell differentiation has been described for *Danio rerio* (Hamilton, 1822) (Hamling et al. 2015), but no studies have addressed this issue for Syngnathidae. We showed that Purkinje cells differentiate as early as 6 DAB in *H. barbouri* and continue to multiply until 35 DAB. This may be necessary to coordinate movements of various body and swimming motions in the larval fish (Kimmel et al. 1995, Hamling et al. 2015). To investigate possible connectivity the cerebellum function and Purkinje cells, further studies using a variety of different labeling techniques will be needed.

In conclusion, our new description of the CNS and brain development in *H. barbouri* provides a foundation for neurobiology and the potential structural basis of the ecology of this seahorse. In particular, the largest optic tectum implies a great capacity for learning and the propensity to feed on fast-moving prey. Another important finding in this study is that the increase in blood vessels in the optic tectum and the saccus vasculosus, as well as the development of the Purkinje cell layer. Since these structures develop at 14 DAB, we speculate that appropriate behavior responses will be observed around this time in *H. Barbouri*, but this hypothesis should be confirmed by future chronology studies on the feeding behavior of this fish.

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